GenomEast Platform
CERBM GIE – IGBMC
1 rue Laurent Fries
67404 Illkirch Cedex
France

+33 3 88 65 34 26
thibault@igbmc.fr
jost@igbmc.fr
genomeast.igbmc.fr

PRODUCT SHEET: QUANTITATIVE RT-PCR

The GenomEast platform offers high-throughput quantitative RT-PCR with Fluidigm technology (https://www.fluidigm.com/products/biomark-hd-system) for gene expression analysis.

1 Fluidigm technology

The BioMark ™ HD System developed by Fluidigm, is an automated real-time PCR equipment that uses microfluidic technology to simultaneously analyze tens of samples in nanoliter scale reactions. This system is compatible with multiple chemistries (Taqman, EvaGreen, SybrGreen, UPL, etc.) and any sample type for various applications. It is thus particularly suitable for simultaneously quantifying the expression of tens of genes in a large number of samples.

PCR reactions are conducted in integrated fluidic circuits (IFC), the Dynamic ™ Arrays. Charging and mixing of primers, samples and reagents in the arrays is automated using an IFC Controller. Due to the small final volume of PCR reactions (6 to 9 nl), it is necessary to increase the concentration of the target genes in each sample prior to loading on the arrays and analysis on the BioMark ™ HD. This **S**pecific **T**arget pre-**A**mplification (STA) consists of a multiplex PCR with a reduced number of cycles (10 to 20 cycles) using a pool of all primers pairs or probes that will be used later on the BioMark ™ HD.

	Flex Six IFC	48.48 IFC	96.96 IFC
			
Number of "sample" wells	6x12	48	96
Number of "primer" wells	6x12	48	96
Number of PCR reactions	864	2304	9216
Note	Ability to execute the 6 partitions of 12x12 independently within 3 months.		

Table 1: IFC Formats available on the GenomEast platform.

The "Real-time PCR analysis User Guide" (PN 68000088), available on Fluidigm's website, provides all the necessary information about the analysis software and the real-time PCR protocols used on the BioMark ™ HD.

2 Experimental design

In order to facilitate the evaluation of the quality and the interpretation of the final results, it is recommended to reserve several "sample" and "primer" wells on each Dynamic ™ Array for control points.

In addition, because of the multiplex PCR reaction at the STA stage, it is difficult to predict the behavior of the primers selected for the assay. Consequently, it is recommended to provide an additional array for the

GenomEast Platform
CERBM GIE – IGBMC
1 rue Laurent Fries
67404 Illkirch Cedex
France

+33 3 88 65 34 26
thibault@igbmc.fr
jost@igbmc.fr
genomeast.igbmc.fr

validation of primers and pre-amplification conditions, on one or more known reference samples that can be deposited at several serial dilutions.

2.1 Controls in « Sample » wells

- Provide at least one well for a negative control on the array at the real-time PCR step. The possible controls are :
  - Blanck = only TE buffer in the chamber
  - NAC= No Amplification Control = Everything but Taq polymerase
  - NTC= No Template Control = Everything but sample
- Provide 6 to 8 wells for a calibration curve made with serial ½ dilutions of the same sample to verify PCR primers efficiency.
  - This curve can be made from a specific reference sample or from the pool of all cDNAs distributed on the array.
- In a project using multiple arrays, provide a minimum of 3 wells per array for inter-array calibrators that will be loaded on all arrays to normalize the intensity between arrays independently of the test samples.
  - For example, it is wise to repeat 3 points of the calibration curve above on all arrays using the same cDNA stock. The amount of cDNA control prepared must then be sufficient for all future arrays and this cDNA should be aliquoted to limit the freezing / thawing cycles to 2 maximum and stored at -80 ° C.
- In addition to including biological replicates for each experimental group in order to assess biological variability, it is strongly advised to perform the reverse transcription reaction at least in duplicate on each sample.

2.2 Controls in « Primer » wells

- Provide at least one well for a negative control on the array at the real-time PCR step (NRC = No reagent Control = No primer)
- Provide a minimum of 3 or 4 primer pairs or probes for housekeeping genes, if possible with expression levels equivalent to those of the target genes interrogated.

3 Services provided

1. Starting RNA samples checking :
   - Quantification using a fluorimeter (Qubit ou Varioskan, Thermofisher) and quality control by capillary electrophoresis (Bioanalyzer, Agilent).
   - This verification is conducted by sampling on a batch of 12 total RNAs randomly selected on the 96-well plate.
   - All samples can be validated for an additional cost on request at the time of the project submission.

2. Reverse transcription of total RNA in cDNA in a total volume of 5 µl

3. The specific target pre-amplification by multiplex PCR with a reduced number of cycles (10 to 20).

4. When using Evagreen chemistry, the treatment of pre-amplified amplicons with exonuclease to remove unused primers.

5. Priming the Dynamic ™ array and loading it with pre-amplified amplicons and primer pairs or Taqman probes using the appropriate IFC controller.

GenomEast Platform
CERBM GIE – IGBMC
1 rue Laurent Fries
67404 Illkirch Cedex
France

+33 3 88 65 34 26
thibault@igbmc.fr
jost@igbmc.fr
genomeast.igbmc.fr

6. Real time PCR on Biomark™ HD.

7. The generation of Ct values using Fluidigm « Real-Time PCR Analysis Software ».
   - This software is freely accessible at https://www.fluidigm.com/software (Package « Biomark & EP1 Software »).
   - This tool allows you to visualize individual amplification and melting curves and eventually to re-analyse the data using different settings.

8. Downstream data analysis (optional, see section 7 for more information).

4 Starting samples and primers to be supplied by users

4.1 Characteristics of total RNAs

Users must provide all total RNA samples at an identical concentration in a 96-well plate with a plate map in Excel format.

Since samples will be handled with 8-channel pipettes, if the 96-well plate provided is incomplete, it is required that the RNAs be organized by 8-well column and not by 12-well line on the plate.

.

Characteristics of total RNA to be supplied	
Optimal concentration	25 to 100 ng/µl
Optimal volume	4 to 10 µl/well
Optimal quality	RIN ≥ 7 and absence of genomic DNA contamination on a Bioanalyzer profile (Agilent)
Quantity required for quantitative validation by fluorimetry	5 to 200 ng of RNA per sample
Quantity required for qualitative validation by capillary electrophoresis	5 to 200 ng of RNA per sample
Quantity required by RT reaction *	2 ng to 250 ng, provide 10 to 14 pre-amplification cycles 2.5 pg to 2 ng, provide 15 to 20 pre-amplification cycles
Total volume per RT reaction	1 to 4 µl

** Based on our past experience, we recommend using 50 to 100 ng starting total RNA per reaction with 14 pre-amplification cycles. The quantities indicated in this table correspond to Fluidigm's recommendations. It may be necessary to provide a preliminary experiment to determine the amounts of RNA and optimal pre-amplification conditions.*

4.2 Characteristics of primers or probes

Users must design the primers or Taqman probes themselves and transfer them to the platform at the same time as their samples.

GenomEast Platform
CERBM GIE – IGBMC
1 rue Laurent Fries
67404 Illkirch Cedex
France

+33 3 88 65 34 26
thibault@igbmc.fr
jost@igbmc.fr
genomeast.igbmc.fr

Characteristics of the primers or probes to be supplied	
Recommendations for primer design	Tm : 60–63°C ; ≤3°C difference between the various primers used on the array GC content : < 50% Length : 18–30 nucleotides Residue G or C at the 3 'end of the primers Length of amplicons: 70 to 200 bp Amplicons should, if possible, cross an intron to avoid amplification of genomic DNA Avoid self-complementarity between primers to reduce the risk of dimer formation
Concentration	***Taqman chemistry***: 20X mixture composed of unlabeled PCR primers and TaqMan® MGB probe (FAM-MGB or VIC-MGB) ***Evagreen chemistry***: pair of sense and antisense primers each at a final concentration of 100 µM
Total volume	10 to 15 µl per primer pair or probe

5 Quality controls

The nature and number of quality controls performed by the platform depend on the initial planning of the experiment.

The checking of primer/probe specificity (i.e. concordance between measured and expected Tm, absence of secondary amplification products ...) is the responsibility of the project leader.

1. Starting samples checking	
Quantity (Fluorimetry)	≥ minimal required quantity <30% difference in concentration between the different verified samples
Quality (capillary electrophoresis)	RIN ≥ 7
2. Q-PCR checking	
Negative controls	Absence of signal in wells without DNA, without enzyme and / or without primer / probe
ROX passive reference (present in the PCR Master Mix)	Verification of the homogeneous distribution of the reaction mixture in all the chambers of the array
Positive controls	Linearity of the calibration curve on the reference cDNA Detection of housekeeping genes in the optimal CT range: 6 to 25
Technical RT replicas	<0.5 CT difference between replicas

6 Results delivery

For each array, the following results are made available to the project manager via an ftp server:

- Raw Q-PCR data from BioMark ™ HD for data analysis and visualization using Fluidigm « Real-Time-PCR analysis Software ».
- Heat-map representation of Q-PCR results.
- Loading image of passive ROX reference in all chambers of the array.
- Table results of CT values and quality scores in csv format.
- Copy of all protocols used as well as plate maps for samples and primers.

GenomEast Platform
CERBM GIE – IGBMC
1 rue Laurent Fries
67404 Illkirch Cedex
France

+33 3 88 65 34 26
thibault@igbmc.fr
jost@igbmc.fr
genomeast.igbmc.fr

According to the "GenomEast Platform terms and conditions of business", the project manager is responsible for his data to be saved and archived on its own. Following their transfer to the Beneficiary, the Platform guarantees the conservation of raw data only for a limited period of six months.

7 Downstream analysis (optional)

Downstream data analysis is not part of the standard service but can be done in collaboration between the project manager and the platform. The following analyses may be performed:

- Inter-plate standardization
- Calculation of ΔCt
- Statistical analysis of expression results

This list is not exhaustive and we recommend the project manager who would like to collaborate with the platform for data analysis to contact the platform before starting their experiment so that we can define the analyses that best fit to the project manager's needs.